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Ara h 2: crystal structure and IgE binding distinguish two sub-populations of peanut allergic patients by epitope diversity

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Abstract

Background—Peanut allergy affects 1% of the population and causes the most fatal food-related anaphylactic reactions. The protein Ara h 2 is the most potent peanut allergen recognized by 80–90% of peanut allergic patients.

Methods—The crystal structure of the major peanut allergen Ara h 2 was determined for the first time at 2.7 Å resolution using a customized MBP-fusion system. IgE antibody binding to the MBP fusion construct versus the natural allergen was compared by ELISA using sera from peanut allergic patients.

Results—The structure of Ara h 2 is a five helix bundle held together by four disulfide bonds and related to the prolamin protein superfamily. The fold is most similar to other amylase and trypsin inhibitors. The MBP-Ara h 2 fusion construct was positively recognized by IgE from 76% of allergic patients (25/33). Two populations of patients could be identified. Sub-population 1 (n=14) showed an excellent correlation of IgE antibody binding to natural versus recombinant Ara h 2. Sub-population 2 (n=15) showed significantly reduced IgE binding to the MBP fusion protein. Interestingly, about 20% of the IgE binding in sub-population 2 could be recovered by increasing the distance between MBP and Ara h 2 in a second construct.

Discussion—The reduced IgE binding to the MBP-Ara h 2 of sub-population 2 indicates that the MBP molecule protects an immunodominant epitope region near the first helix of Ara h 2. Residues involved in the epitope(s) are suggested by the crystal structure. The MBP-Ara h 2 fusion constructs will be useful to further elucidate the relevance of certain epitopes to peanut allergy.

Keywords

Peanut; Allergy; Ara h 2; Immunotherapy; Structure

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Contributions: GAM conceived of the study, performed structural analysis, and wrote the manuscript. RAG refined the crystallographic coordinates. AP and SW designed and executed the IgE studies and analysis. AFM contributed to the production of sample. REL conceived of the study and edited the manuscript. LCP produced the sample, collected data, and refined the coordinates. All authors contributed to the editing of the document.

Introduction

The prevalence of food allergy is estimated to be 6% in young children and 3.7% in adults (1). Most children grow out of common allergies to milk or eggs, but allergies to peanuts generally persist, affecting approximately 1% of the population (2). Peanut allergies are of particular concern due to the extreme hypersensitivity of some individuals (less than 100 μg dose (3)) and adverse reactions to peanuts are the most frequent type of fatal anaphylaxis among food allergens (4). Ara h 2 is the most potent peanut allergen recognized by >90% of peanut allergic patients (5–7). Studies in children demonstrated that Ara h 2 and the homologous Ara h 6 (59% identity) are the most commonly recognized allergens and IgE reactivity to these proteins is a risk factor for the most serious reactions (8,9).

Currently, patients are advised to strictly avoid peanut consumption. In traditional immunotherapy treatments for allergy, patients are exposed to small but escalating doses of protein (10). Studies with peanuts have demonstrated initial promise, but still use extremely small doses of peanut protein in order to avoid serious side effects and, at present, utilize only oral administration due to safety concerns (10,11). It has been proposed that a safer alternative would be to design hypoallergenic variants of the major allergens, which could avoid the serious side effects, allow for higher doses, and still generate tolerance or desensitization (5,12). There have been many attempts to modify inhalant and food allergens (12,13), however this approach seems particularly appropriate for peanut allergy since the adverse reactions can be severe.

Herein, we present the first empirically determined crystal structure of Ara h 2 at 2.7 \AA , which we have used to analyze IgE antibody binding using sera from peanut allergic patients. Antibody epitopes usually extend 600–900 \AA^2 in surface area and, except in special cases, interact with discontinuous elements of the primary structure (14). Indeed, Albrecht et al demonstrated that peptides derived from Ara h 2 could not inhibit IgE binding to the native allergen, and unfolded Ara h 2 had significantly reduced IgE binding capacity (15). While mapping antibody epitopes with peptides is expedient and may provide some useful information, the full structure can provide detailed information about the complete interacting surface.

Materials and Methods

Crystallization and Structure Determination

A codon-optimized gene of Ara h 2.01 was obtained from GenScript (Piscataway, NJ) and used as a template for PCR to amplify the DNA to be inserted into the pMALX_E plasmid (16) using the NotI and EcoRI restriction sites. The pMALX_E plasmid contains the MBP mutations D82A, K83A, E172A, N173A, K239A, and E359A as well as changes in the C-terminal helix as previously described to improve the likelihood of crystallization (16). Several constructs with different N-terminal truncations of Ara h 2 were tested for expression of soluble protein (data not shown). The two constructs discussed in this paper are rMBP-Ara h 2-N19 and rMBP-Ara h 2-N28, which connect to the C-terminus of MBP-pMALX_E via the N-terminus of Ara h 2 residues 19 or 28, respectively, following the numbering of Stanley et al (5). Origami B cells were serially transformed with a pACYCDuet-1 plasmid encoding thioredoxin, followed by the pMALX_E Ara h 2 plasmids. For large scale purification, cells were grown in 12 L of LB media containing 100 mg/ml ampicillin, 35 mg/ml chloramphenicol, 12.5 mg/ml tetracycline, and 50 mg/ml kanamycin in twelve 2.8 L Fernbach flasks at 37° C. When the A_{600} reached 0.75, the temperature of the incubator was set to 18° C. When the incubator temperature reached 18° C, IPTG was added to a final concentration of 500 μM and the cells were allowed to grow overnight. Cells were pelleted by centrifugation and lysed by sonication in 25 mM Tris pH 7.5, 500 mM NaCl and

1 mM EDTA containing Complete protease inhibitors (Roche). Soluble protein was separated from debris by centrifugation and loaded in batch onto amylose resin at 4° C. The resin was washed with sonication buffer followed by elution with sonication buffer containing 40 mM maltose. Protein was concentrated then loaded onto a 16/60 Superdex200 column pre-equilibrated with elution buffer. Peaks containing pure MBP-Ara h 2 protein were pooled and dialyzed against 25 mM Tris pH 7.5, 75mM NaCl, and 5mM maltose. Protein was then concentrated to 38 mg/ml and used for crystallization trials.

Diffraction quality crystals of MBP-Ara h 2-N28 were obtained using the vapor diffusion hanging drop method, mixing 1 µl of protein with 1 µl of reservoir containing 0.1 M sodium citrate pH 5.5 and 1.7 M ammonium sulfate. Resulting crystals were used for micro-seeding into similar conditions containing a reservoir of 0.1 M sodium citrate pH 6.5 and 1.8 M of ammonium sulfate. For data collection, a small, thin plate was transferred to 0.1M sodium citrate pH 6.5, 100 mM NaCl, 2.2 M ammonium sulfate, 5 mM maltose, and 15% ethylene glycol and then flash frozen in liquid nitrogen. Data were collected at APS to 2.7 Å on the ID22 SER-CAT beamline. Data were processed using HKL2000 (17). CCP4i was used to obtain initial phases (18). MolRep (19) was used to determine the position of the MBP using coordinates from pdb code 2DMO followed by initial refinement in Refmac (20). Coot was used to build Ara h 2 into the electron density (21). The structure of Ara h 6 was manually placed into the electron density and used as a visual guide for model building. The structure was further refined through iterative cycles of model building in Coot and refinement in Phenix (21,22). All MBP residues are present in the density, as well as residues 28-56 and 84-148 of Ara h 2. Note that in the PDB file (3OB4), the Ara h 2 residues are numbered 1028–1148 to differentiate them from the N-terminal MBP fusion. In addition, density for eleven residues found in a lattice contact with an MBP molecule have been modeled with residues 59-68 of Ara h 2. The overall quality of the model has been assessed using MolProbity and is in the 97th percentile for structures at 2.7 Å +/- 0.25 Å (Table 1) (23).

ELISA for measuring IgE antibodies to Ara h 2

IgE binding to natural Ara h 2 and the recombinant constructs rMBP-Ara h 2-N19 and rMBP-Ara h 2-N28 was analyzed by a chimeric ELISA previously described (24). The plates were coated overnight at 4°C with anti-Ara h 2 mAb 1C4. Plates were blocked with PBS-Tween-1% BSA, and in the following step, they were incubated with 5 mg/mL of either natural Ara h 2 or one of the two recombinant allergen constructs (100 µl per well) at room temperature. Natural Ara h 2 was purified from defatted peanut extract by affinity chromatography using the Ara h 2 specific mAb 1C4. Proteins were quantified by Advance Protein Assay (Cytoskeleton, Denver, CO). Sera from peanut allergic patients (CAP values from 0 to >100 kUA/L; average 26 ± 33 kUA/L for n = 21) were obtained from Bioreclamation (Westbury, NY), which operates in full compliance with Food and Drug Administration guidelines, or kindly provided by Dr. Peter Heymann (University of Virginia). Thirty-three sera were added at 1:2 and 1:10 dilutions, or further diluted for IgE antibody binding to fall in the linear range of the standard curves. Two sera without peanut reactivity were used as negative controls. Anti-Der p 2 mAb αDpX, purified natural Der p 2 at 500 ng/ml, and chimeric Ab 2B12-IgE were used to perform IgE standard curves. Bound IgE was detected using biotinylated goat anti-human IgE (KPL, Gaithersburg, MD) at 1:1,000 dilution. Streptavidin peroxidase was added at 250 ng/ml, followed by development with 1mM ABTS and 0.03% hydrogen peroxide as substrate. IgE antibody binding was quantified by measuring the OD at 405 nm. OD values for negative control sera were subtracted from the values for peanut allergic sera. Relative values of IgE binding were calculated by multiplying OD 405 nm by the dilution at which the sera were tested. As an aside, CD spectra of the two rAra h 2-MBP constructs are nearly identical within

experimental error (data not shown), indicating that Ara h 2 is similarly folded in both constructs.

Results

Ara h 2 was crystallized by the MBP fusion/surface entropy reduction technique that uses an engineered MBP as a carrier protein (Figure 1), to aid in crystal lattice formation (16). The construct that crystallized was rMBP-Ara h 2-N28, which eliminated the 21 residue signal sequence and 6 residues predicted to be disordered based on the NMR structure of Ara h 6 (25). Ara h 2 has five helices and four disulfide bonds with at least one disulfide bond connecting each helix to another (orange arrows Figure 1). The fold of Ara h 2 belongs to the prolamin protein superfamily, which usually consists of a 4–5 helix bundle held together by 4–5 disulfide bonds. Figure 2 shows superpositions of Ara h 2 with other members of the prolamin family. Using the web server DALI (26), the structure of Ara h 2 was ranked most similar to the proteinase/alpha-amylase inhibitors with very high Z scores ranging from 8 to 9.3. The top match is an alpha-amylase inhibitor (AI, Figure 2A) from wheat kernel (27), the second best match is the corn hageman factor inhibitor (CHFI, Figure 2B) (28), and the third best match is the RAGI-bifunctional inhibitor (RBI, Figure 2C) from Indian finger millet (29,30). A significant structural difference between Ara h 2 and RBI or CHFI, is that the bifunctional inhibitors have a short fourth helix interrupted by a structured extended loop (asterisk in Figure 2) that interacts with the target amylase. Ara h 2 and AI have a longer helix ($\alpha 4$) in a structurally similar position (Figure 2A), so Ara h 2 is structurally most similar to AI.

Other structural matches to Ara h 2 include Ara h 6, which is the fourth highest Z-score (Figure 2D) and has the highest sequence identity (59%). Previous studies have also compared Ara h 2 with the 2S albumins such as SFA-8 from sunflower (Figure 2E) and the castor bean allergen Ric c 3 (31). However, the low DALI scores clarify that these are more distantly related. Additionally, the lower Z-scores and a visual inspection confirm that Ara h 2 is more similar to the proteinase/alpha-amylase inhibitors and the 2S albumins, than it is to the nsLTP family as shown with the example in Figure 2F (32).

The extended 31 residue loop between helices 2 and 3 of Ara h 2 was demonstrated to be sensitive to proteolysis (25) and is mostly unobserved in the crystal structure. The loop may also be cleaved in the crystal, however, mass spectrometry of the sample used for the crystallization found no evidence of proteolysis (data not shown) suggesting that the residues are simply disordered. The few residues of this loop that are observed (orange color Figure 1) are significantly displaced from comparable residues in Ara h 6, but this region is not well conserved and is known to be flexible (25). The sequence of this disordered loop differentiates Ara h 2.01 and Ara h 2.02, hence the structure here can be considered a model for the core structure of either isoform of Ara h 2.

In order to assess the correct folding of Ara h 2 in the MBP fusion construct, IgE antibody binding to the natural allergen was compared to the recombinant constructs (Figure 3). Twenty-nine of thirty-three sera samples from peanut allergic patients reacted to natural Ara h 2, representing an 88% (29/33) prevalence of IgE antibody binding to this allergen. The overall percentage of sera reacting to rMBP-Ara h 2-N28 was 76% (25/33) with a clear distinction between two sub-populations that had previously reacted positively to the natural allergen. Sub-population 1 (n=14) showed an excellent correlation of IgE antibody binding to natural versus the rMBP-Ara h 2-N28 construct, indicating the recombinant Ara h 2 used in this crystallographic study is properly folded (Figure 3A and 3C). In contrast, sub-population 2 (n=15) showed low IgE antibody binding to rMBP-Ara h 2-N28 with an average binding reduction of 90% compared to the natural allergen. To test whether the

MBP molecule was protecting an important IgE epitope region, the IgE antibody binding of 15 sera from subpopulation 2 was tested against rMBP-Ara h 2-N19, a construct with 9 more amino acids between MBP and the first helix of Ara h 2 (Figure 3B). The IgE antibody binding was on average 19% higher to the rMBP-Ara h 2-N19 than the binding to the rMBP-Ara h 2-N28 construct (Figure 3B and 3C). The construct with longer linker between the molecules should allow more access of IgE to the protected epitope(s). Additionally, the range of relative IgE antibody binding (OD 405 nm times sera dilution) was similar for natural and rMBP-Ara h 2-N28 allergen for sub-population 1 (mean absorbance of 6.0 ± 11.9 and 6.7 ± 14.0 OD units, respectively). In contrast, for sub-population 2, the range was larger for IgE antibody binding to natural allergen (mean absorbance of 91.3 ± 88.1) than for rMBP-Ara h 2-N28 (5.3 ± 4.8) or rMBP-Ara h 2-N19 (20.1 ± 18.6) (Figure 3C).

In examining the structure of the fusion protein for residues likely to be blocked by MBP-Ara h 2-N28, residues were classified into three categories using different sized spherical probes to estimate access to Ara h 2 by IgE. The radii of the probes were: 1.4 Å (similar to solvent and very likely obscured by MBP), 10 Å (an intermediate value), and 17 Å (a circle with equivalent radius would be similar in size to an antibody epitope). Figure 4 shows the residues colored by estimated relative accessibility according to the spherical probes. The same color scheme is reiterated in Figure 5 to specify exactly what residues are potentially protected by MBP. Broadly speaking, it is very likely that residues in helix 1 are obscured by MBP, moderately likely that residues in helix 4 are obscured by MBP, and possible that residues at the C-terminus and in helix 3 would be difficult to access with a bulky antibody based on this analysis. It is important to state that this analysis is extremely qualitative and it is unknown if there is any flexibility between MBP and Ara h 2 in solution.

Discussion

The fold of Ara h 2 belongs to the prolamin superfamily, which is subdivided into three families (33), the non-specific lipid transfer proteins (nsLTP), the proteinase/alpha-amylase inhibitors, and the seed storage 2S albumins. The nsLTP's have a hydrophobic cleft for binding lipid substrates, which is not present in Ara h 2. Based on the structural data presented here, Ara h 2 belongs in the proteinase/alpha-amylase inhibitor family. The initial motivation for the development of the MBP constructs that are tightly packed against Ara h 2 was to aid in crystallization. To date, 21 different proteins have been successfully crystallized using MBP as a carrier protein (16). The MBP fixed-arm constructs, such as the MBP-Ara h 2, have been used to solve the structures of other unrelated proteins, including another allergen, Der p 7 (34). There are two main lines of evidence that suggest the structure of Ara h 2 determined here is not significantly perturbed by MBP. First, the structure is similar to other members of the prolamin family (Figure 2) including the proper connectivity of the disulfide bonds, as predicted from sequence homology (31). And second, in 42% (14/33) of the sera from peanut allergic patients there is excellent correlation between IgE binding to the natural allergen and the fusion protein indicating that MBP does not alter the overall structure (Figure 3).

Additionally, the MBP-Ara h 2 fusion constructs have proven useful tools for identifying areas in Ara h 2 protected from antibody access by MBP. These protected areas appears to contain immunodominant IgE epitopes for a sub-population of peanut allergic patients. In this sera sub-population 2 (n=15), the IgE antibody binding to rMBP-Ara h 2-N28 was strongly reduced (by 90%) compared to the natural allergen, and the correlation between IgE antibody binding to both allergens was low ($r = 0.13$) (Figure 3C). Interestingly, with a longer linker (construct rMBP-Ara h 2-N19) a fraction of the patient response (19%) could be recovered and the correlation between IgE antibody binding to natural versus the construct rMBP-Ara h 2-N19 is positive ($r = 0.61$) compared to the rMBP-Ara h 2-N28

construct. From these results, MBP appears to block IgE antibody binding to an immunodominant epitope region for the set of sera from sub-population 2, rather than altering the rAra h 2 structure. In agreement with these results, one of the previously reported immunodominant linear epitopes of Ara h 2 corresponded to residues 27-36 (5), which is significantly blocked by MBP. It is intriguing that sub-population 2 appears to have a main immunodominant epitope region near helix 1 (Figures 4 and 5). A positive correlation between the number of IgE epitopes recognized and clinical sensitivity has been reported (35, 36). Therefore, the use of MBP-Ara h 2 fusion constructs in future studies should continue to elucidate the relevance of these Ara h 2 epitopes in peanut allergy.

The structure also provides information of potential value for the design of possible hypoallergenic mutants as described below. Hypoallergens seek to improve the safety and efficacy of immunotherapy by reducing IgE binding while maintaining important T-cell epitopes (5,11,12,37). Figure 5 graphs the fractional surface exposure of each residue, and highlights in magenta important residues for IgE binding as identified with peptide mapping (5). These studies suggested that residues R28, R29, Q31 and E35 were crucial for IgE binding based on alanine substitutions (5). All of these residues are significantly surface exposed in the structure so the alanine scanning nicely matches the relative surface area for the residues where there is structural data. Based on the mapping, a mutant Ara h 2 with multiple residues changed to alanine (arrows Figure 5) was created that demonstrated reduced IgE binding (12). The structure presented here indicates that the mutations P41A, L130A, and L148A are likely of limited value in reducing IgE binding because of low surface exposure, whereas the highly solvated E35 and D53 may represent important antigenic features for IgE binding. Based on Figure 5, future design could focus on surface residues such as Q48, Q51, R52, E86, E111, Q135, Q143, R144, D146, and/or D148 for mutational studies to reduce IgE binding. These mutations avoid key T-cell epitopes (Fig. 5 black box), thought to be pivotal for the efficacy of selective allergen immunotherapy (11,37). We anticipate that the structural data presented here will provide useful information for the rational design of hypoallergenic Ara h 2 molecules.

Additional bioinformatic analysis of epitopes from the structure and comparisons of Ara h 2 and possible Ara h 6 epitopes can be found in the Supplementary Material.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

MBP maltose binding protein

RMSD root mean squared deviation

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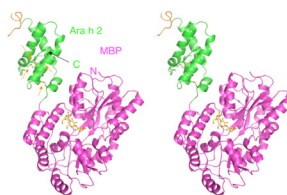


Figure 1. Stereo view of MBP-Ara h 2 fusion protein. The colors are: Ara h 2 (green and orange (residues 56-69)), MBP (magenta), maltotriose (yellow). Orange arrows indicate the disulfide bonds.

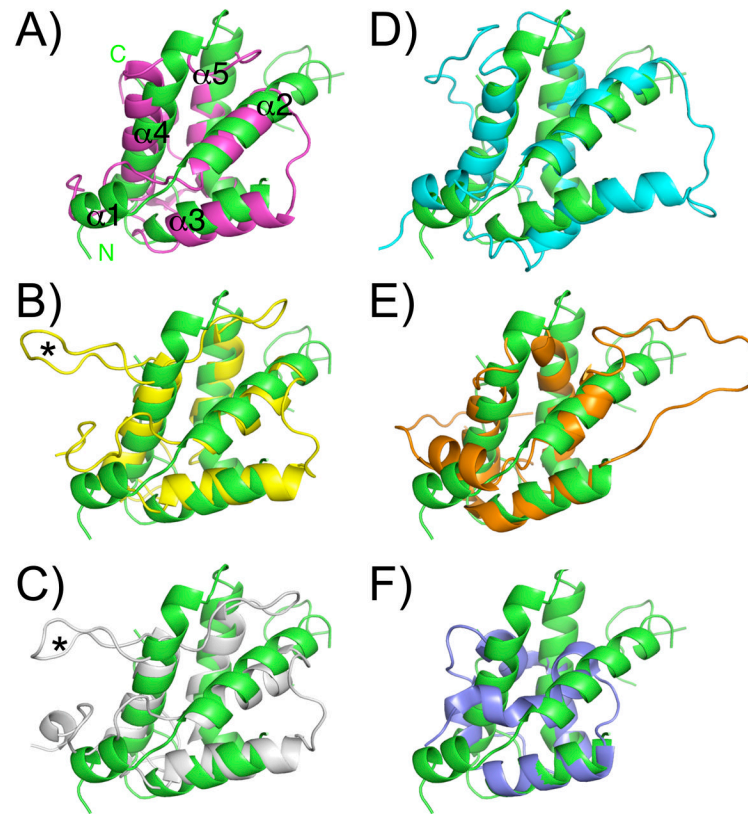
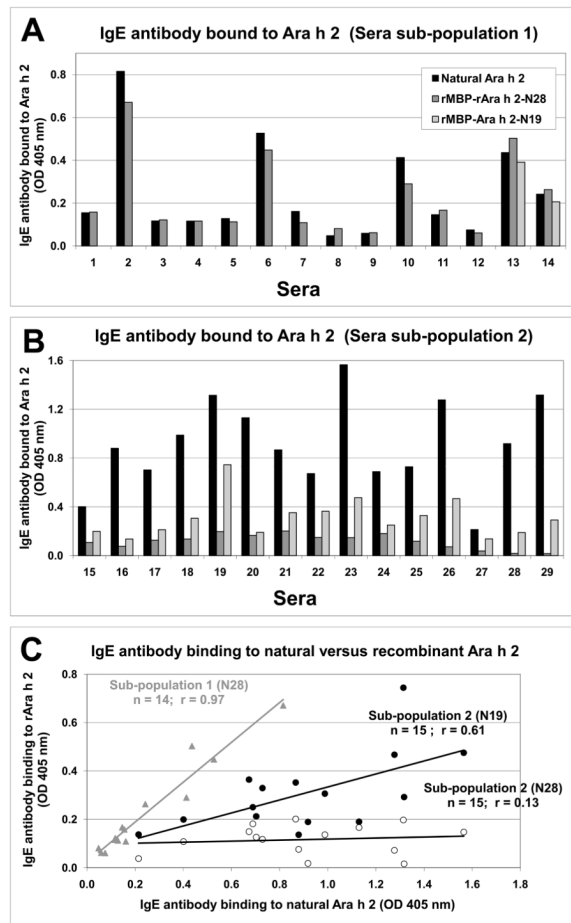


Figure 2. Superpositions of Ara h 2 and other prolamin family members. Ara h 2 is green in all panels. A) AI, magenta B) CHFI, yellow C) RBI, white D) Ara h 6, cyan E) SFA-8, orange F) nsLTP, blue. The asterisk indicates a structured loop in the bifunctional inhibitors RBI and CHFI that interacts with an amylase.

**Figure 3.**

IgE binding to MBP-Ara h 2 fusion constructs and natural Ara h 2 measured by ELISA. A) IgE antibody binding to natural and recombinant Ara h 2 using 14 sera in sub-population 1. B) IgE antibody binding to natural and recombinant Ara h 2 using sera from sub-population 2. Sera #26–29 were considered negative for IgE binding to rMBP-Ara h 2-N28 due to values below the non-allergic control. In panels A & B, black bars represent natural Ara h 2, grey bars rMBP-Ara h 2-N28, and light grey rMBP-Ara h 2-N19. Panel C shows the correlation of IgE antibody binding to natural Ara h 2 versus recombinant fusion constructs. Grey triangles represent sub-population 1 comparing natural versus rMBP-Ara h 2-N28. Open circles show sub-population 2 comparing natural versus rMBP-Ara h 2-N28. Filled circles represent sub-population 2 comparing natural versus rMBP-Ara h 2-N19. Results are reported as OD 405 nm measured for the sera at dilutions that fall in the linear range of the standard curve.

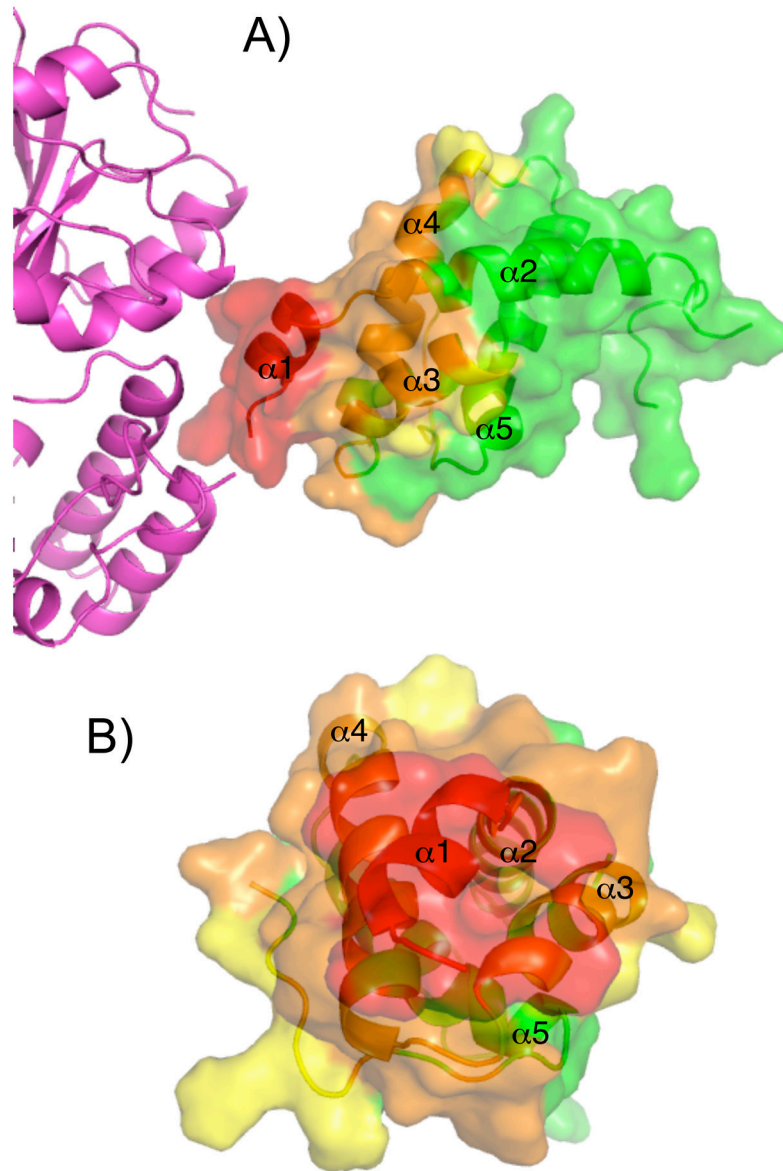


Figure 4. Proposed surface of Ara h 2 that is inaccessible to IgE due to MBP. Spheres of various sizes (1.4, 10, and 17 Å) were used to estimate the relative accessibility of residues. MBP is magenta in panel A and absent in panel B. The surface and ribbon diagram of Ara h 2 are colored green for accessible, yellow for possibly accessible, orange for unlikely to be accessible, and red for most likely to be inaccessible. The perspective in panel B is looking from the MBP molecule.

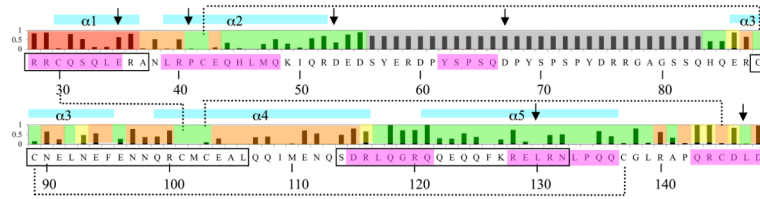


Figure 5.

Ara h 2 Epitope Data and Fractional Side Chain Accessibility. Black bars plot the fractional side chain accessibility determined by VADAR (38). Overlaid on the plot, residues that are inaccessible due to MBP measured with a 1.4, 10, and 17 Å sphere are highlighted red, orange, and yellow, respectively. Other residues are highlighted green. Other highlights indicate: disordered residues and estimated side chain accessibility (grey); Helices (cyan); potentially important peptide IgE epitopes in (5) (magenta); T-cell epitopes (37) (black box). Arrows indicate the positions of a multiple alanine-substituted mutant of Ara h 2 with reduced IgE binding (12). Dotted lines indicate disulfide bonds.

Table 1

Crystallographic data statistics

| | |
|--|--|
| data set | MBP-Ara h 2 (28–148) |
| unit cell | a=68.85Å, b=87.38Å, c=113.14Å; $\alpha=\gamma=90^\circ$, $\beta=103.93^\circ$ |
| Space Group | C2 |
| Resolution (Å) | 50.0 - 2.7 |
| # of observations | 56,256 |
| unique reflections | 16,353 |
| Rsym(%) (last shell) ¹ | 10.4 (32.1) |
| I/ σ I (last shell) | 7.9 (2.3) |
| Mosaicity range | 0.55–0.95 |
| completeness(%) (last shell) | 91.7 (50.0) |
| <u>Refinement statistics</u> | |
| Rcryst(%) ² | 18.3 |
| Rfree(%) ³ | 25.6 |
| # of waters | 69 |
| Overall Mean B value (Å) | 39.8 |
| Average for MBP | 37.8 |
| Ara h 2 | 47.0 |
| maltotriose | 26.1 |
| water | 31.6 |
| <u>r.m.s. deviation from ideal values</u> | |
| bond length (Å) | 0.005 |
| bond angle (°) | 0.8 |
| dihedral angle (°) | 18.7 |
| <u>Ramachandran Statistics⁴</u> | |
| residues in: | |
| favored (98%) regions (%) | 95.5 |
| allowed (>99.8%) regions (%) | 99.8 |
| MolProbity Score | 2.01, 97 th percentile |

¹ $R_{\text{sym}} = \sum(|I_i - \langle I \rangle|) / \sum(I_i)$ where I_i is the intensity of the i th observation and $\langle I \rangle$ is the mean intensity of the reflection.

² $R_{\text{cryst}} = \sum||F_o| - |F_c|| / \sum|F_o|$ calculated from working data set.

³ R_{free} was calculated from 5% of data randomly chosen not to be included in refinement.

⁴ Ramachandran results were determined by MolProbity.